Electrophoretic Pattern of Amylase Isoenzymes in Serum and Urine of Normal Persons

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We separated and measured amylase isoenzymes in the serum and urine of 3036 normal persons by electrophoresis on a thin layer of polyacrylamide gel. We wished to establish the normal pattern of these isoenzymes and to evaluate the usefulness of this method of electrophoresis in clinical diagnosis. Results for patients with hyper- or hypofunctioning pancreas and salivary glands suggested that essentially all the isoamylases in human serum and urine are derived from the salivary glands and the pancreas, and revealed that isoamylases of more than 98% of normal persons consisted of two major isoenzymes and two to three minor ones. Although these observations indicate that data on changes in the proportion of amylase activity of each isoenzyme can be useful in clinical medicine, the following points should be remembered: (a) quantitative differences in the isoenzyme pattern were observed, depending upon the condition of the samples; (b) because the proportion of isoenzyme activity in serum of different normal persons differs, seriatim determination of amylase isoenzymes is necessary; and (c) because five different genetically controlled types of isoenzymes were observed in normal persons, genetic investigations are also necessary.

Additional Keyphrases: electrophoresis on polyacrylamide gel • normal values • origin of serum amylase • diagnosis of pancreatitis • genetics • isoenzyme pattern in infants • interindividual variation

Measurement of total serum amylase activity is used in the diagnosis of acute pancreatitis, the first enzymatic method to be used in clinical medicine (1). Abnormally high amylase activities in serum have been reported in a large variety of conditions (2–5), and thus a more accurate diagnosis should result if one separates the amylase isoenzymes, identifies the source of each, and ascertains which isoenzymes are related to which disease.

Such a separation is now possible as one of the routine tests in many laboratories (6–22), but the normal pattern of amylase isoenzymes in serum and urine has not yet been established.

We determined amylase isoenzymes in serum and urine of 3036 normal persons by electrophoresis on polyacrylamide gel, in an attempt to establish the normal pattern of amylase isoenzymes. Furthermore, the usefulness of this method in clinical medicine was investigated.

Materials and Methods

Samples

The types of samples we examined included saliva, pancreatic juice, urine, serum, and homogenates of human pancreas, parotid glands, and liver. Whole human saliva was collected sublingually, without preliminary stimulation, by continuous aspiration into a collecting tube, and centrifuged to remove particulates. Pancreatic juice was obtained at surgery by intubation of the pancreatic duct. The urine used was the first morning specimen, collected from 3036 students, 20 to 25 years old and without known diseases. Serum was collected before breakfast from 750 students 22–25 years old and 150 additional samples from apparently normal children 0–13 years old. The samples from patients included five cases of mumps, 10 of acute pancreatitis, three cases of decreased amylase activity of saliva, a case of complete pancreatectomy, and a case of chronic relapsing pancreatitis. Specimens of pancreas, parotid glands, and liver were obtained within 3 h after death of patients who had died of myocardial infarction. The tissues were freed of connective tissue, washed with distilled water, and homogenized at 4 °C in three volumes of a sucrose solution (0.25 mol/liter) containing CaCl₂ (20 mmol/liter), in a Potter–Elvenhjem homogenizer with Teflon pestle. The homogenates were centrifuged (27 000 × g, 30 min, 4 °C) and the supernates used for electrophoresis.

Amylase isoenzymes were separated and measured within 48 h of specimen collection; storage was at 4 °C for urine, −20 °C for serum.

Procedures

Amylase Isoenzyme Determination

A discontinuous buffer system was used; the gel buffer consisted of 0.19 mol/liter Tris [tris(hydroxymethyl)aminomethane], pH 8.8, the electrode buffer of 0.3 mol/liter borate, pH 8.2.

The monomer-mixture solution was prepared by dissolving acrylamide and bis(N,N'-methylenebisacrylamide) in a proportion by weight of 95 to 5 in gel buffer. As the catalyst, we used ammonium persulfate (1.2 g/liter) and TEMED (N,N'-tetramethyl-ethylene diamine, 70 mmol/liter). Two parts of monomer mixture solution and one part of each of the two catalyst solutions were mixed and poured into the gel mold to make a gel 1 mm in thickness. Four
microliters of the sample was applied in the sample groove of the gel with a micropipet and horizontal electrophoresis was done at 4 °C for 2.5 h at 0.8 mA per centimeter (Toyo Immunelectrophoresis System, Toyo AE-2; Toyo Electrophoresis Stabilizer, PS-310; Toyo Kagaku Sangyo Co. Ltd., Osaka).

After electrophoresis, the gel plate was incubated for 30 min at 37 °C in soluble-starch solution (10 g/liter) containing NaCl (2 g/liter), followed by incubation of the gel plate alone at 37 °C for 15 to 120 min, depending on the amylase activity of the sample. The gel plate was soaked in dilute acetic acid (50 ml/liter) for 5 min and then stained with an iodine/potassium iodide solution (30 g of KI and 13 g of I2 per liter). The area in which amylase activity was present was identified as a light yellow or transparent band against a dark-blue background. The preparations were scanned with a densitometer (Toyo Digital Densitrol, DMU-33C, Toyo Kagaku Sangyo Co.) and the gel plate was printed on a printing paper to get a replica transfer of the electropherogram.

Fundamental Studies on Measurement of Amylase Isoenzymes

**Pre-electrophoresis:** We tested the effect of ammonium persulfate on amylase isoenzymes by removing it by pre-electrophoresis of the polyacrylamide gel before samples were applied.

**Column chromatography:** Column-chromatographic investigations were performed to find out whether or not there were changes in molecular weight during electrophoresis on polyacrylamide gel. The columns for gel filtration were packed according to the instructions of the suppliers of the materials, and all experiments were done at 4 °C. We passed the fresh sample before electrophoresis and the eluates from the gel plate after electrophoresis through a column (2.6 x 90 cm) packed with Sephadex G-75, Superfine (Pharmacia, Uppsala, Sweden). The column was equilibrated and eluted with Tris/maleate buffer (50 mmol/liter, pH 7.0) containing CaCl2 (20 mmol/liter) and NaCl (20 mmol/liter). We collected 4.0-ml fractions and assayed them by a chromogenic method (23) with use of a blue-dyed starch polymer (Pharmacia-Shionogi, Osaka, Japan).

**Comparison of two methods for measuring amylase isoenzymes:** After performing the electrophoresis, we cut the polyacrylamide gel plate into thin slices of 1 mm width, and separately suspended them in 1 ml of phosphate buffer (50 mmol/liter, pH 6.8) at 4 °C for 24 h. Amylase activity so extracted from each slice was measured by both the chromogenic method and the iodometric method.

**Measurement of Total Amylase Activity**

Total amylase activity was measured by a chromogenic method (23) with use of the blue-dyed starch polymer (Pharmacia). Results were expressed as Somogyi units per 100 ml for serum and Somogyi units per hour for urine.

**Results and Discussion**

Our results essentially accord with the findings of others (6–9, 11–22, 24, 25) and confirm the presence of two principal amylase isoenzymes in human serum and urine. However, our methods appear to be more sensitive, because two to three additional isoenzymes were identified. Although Roby et al. (26) reported that during electrophoresis amylase was decomposed into subunits by ammonium persulfate, the strong oxidizing agent used in preparing polyacrylamide gel, the same pattern of amylase isoenzymes was observed whether or not the gel-plate had been pre-electrophoresed. Column chromatographic investigations (Sephadex G-75 gel filtration) revealed no changes in molecular weight during electrophoresis on polyacrylamide gel, though peak 1 was no longer detectable in eluates from the gel after electrophoresis of human pancreatic amylase (Figure 1). This peak accounted for less than 5% of the total pancreatic amylase activity (24) even in the original sample, and no relative corresponding increase in peak 2 was observed; thus, the disappearance of peak 1 from the eluates of the gel after electrophoresis may simply be a reflection of low recovery of total amylase activity.

The possibility of mistaking the amyloclastic activity of other serum proteins as amylase activity (27, 28) was obviated by soaking the gel plate in an acetic acid solution before staining. Furthermore, we satisfied ourselves as to the suitableness of our iodometric assay method by the agreement between patterns obtained with the iodine/starch reaction and those by the chromogenic method (Figure 2). We concluded that the transparent or light-yellow bands against the dark-blue background surely indicate the presence of amylase activity.

**Fig. 1. Results of gel filtration of human pancreatic amylase on a column of Sephadex G-75 (2.6 x 90 cm)**

We used a Tris/maleate buffer (50 mmol/liter, pH 7.0) containing CaCl2 (20 mmol/liter) and NaCl (20 mmol/liter). 4.0-ml fractions were collected. Closed circles: elution pattern of unelecrophoresed human pancreatic amylase; open circles: elution pattern of eluates from the gel after electrophoresis of human pancreatic amylase.
Amylase isoenzymes were then evaluated on the basis of the above studies. Figure 3 shows the results of electrophoretic studies of unmodified serum from the patients mentioned above and of extracts of pancreatic and parotid gland. The patterns of amylase isoenzymes from pancreas and for serum of patients with acute pancreatitis and decreased amylase activity in saliva were the same, but differed from those for saliva and serum of patients with mumps or who had undergone total pancreatectomy. These observations of amylase isoenzyme patterns in hyper- or hypofunctioning states of pancreas or salivary glands would seem to make it possible to specify the origin of the amylase isoenzymes that appear in human serum and urine. However, the existence of extrapancreatic and extrasalivary sources of serum and urinary amylase is still a moot question. Although we could not find any amylase activity in extracts of human liver, Joseph et al. (10) identified an amylase that differed electrophoretically from pancreatic and salivary amylase and proposed its hepatic origin, a hypothesis supported by the observation that amylase activities are decreased in liver diseases (29, 30). Fridhandler et al. (31) reported that amylase activity in liver extracts is too low for it to be a significant source of human serum and urine amylase, and found no isoamylase component peculiar to liver extracts in serum or urine. Finding some differences between serum or urinary amylase and salivary or pancreatic amylase in the degree of inactivation of their activity on heating at 65 °C for 15 min or by trypsin, Warshaw (32) concluded that most amylase activity in normal serum and urine did not originate in either pancreas or salivary glands. Although there were some differences with respect to rate of inactivation of amylase activity by heat at 65 °C for 15 min between serum or urinary amylase and salivary or pancreatic amylase, amylases in all these samples were completely destroyed within 90 min (33). Electrophoretic analysis of heat-treated serum or urine of normal persons revealed (34) that the amylase activity of pancreatic isoenzyme was decreased faster than that of saliva, in agreement with the previous report.

From these observations, it seems difficult to confirm the hypothesis that circulating amylase is neither of pancreatic nor salivary origin.
We attempted, therefore, to make use of the differences in mobility of pancreatic and salivary amylase isoenzymes for diagnosis. It becomes necessary to identify each isoenzyme. Although there are recommendations for the nomenclature of isoenzymes from the IUPAC/IUB International Commission on Enzymes, they seem to us to be unsuitable for amylase isoenzymes because the anodal end of the amylase isoenzyme pattern changes depending upon the amylase activity of the samples, reaction time, or conditions of samples. For example, Figure 4 demonstrates the changes of amylase isoenzymes toward the anode side depending on total amylase activity or on the concentration of bicarbonate in pancreatic juice obtained during pancreozymin/secretin test. The same changes in amylase isoenzymes were observed in serum of patients with acute pancreatitis. Quantitative changes in the isoenzyme pattern have been observed in stored samples (25, 33). Similar changes in the isoenzyme pattern were found in pleural fluids and ascites. Such observations move us to propose that the bands be numbered from cathode to anode, as already is being done by some authors (13, 43, 46). That is, "amylase-1" indicates the amylase isoenzyme with the slowest anodic mobility. It is also premature to designate these isoenzymes as P1, P2 or S1, S2 on the basis of their principal origin (12, 15, 22, 24), because increased amylase activity of the salivary type has been found not only in patients with diseases of salivary glands but also in cases of lung cancer, pneumonia, diabetic coma, and postoperative hyperamylasemia (21, 31, 35–37). With respect to their electrophoretic mobility, amylase isoenzymes 1, 2, 4, and 6, found in serum and urine, showed the same mobility as pancreatic isoamylases, and amylase-3, 5, and 7 the same as salivary isoamylases. Thus, the isoenzymes of the former group are considered the "pancreatic type" and the latter the "salivary type," for the present. The difference in mobility between pancreatic and salivary amylase isoenzymes is slight. Therefore, in case of inadequate separation or of comparative study of different electrophoretic strips as in previous studies, amylase-2 and amylase-3 are apt to be taken as one constituent, although they differ from each other and originate from the pancreas and the salivary gland, respectively. The inadequate methodology for separating these isoenzymes may cause important errors in interpretation of the data obtained and the results. For these reasons, we believe that the densitometric method is incompletely satisfactory.

Total serum and urinary amylase activity of normal infants up to 4 months was below the so-called normal range. Densitometric analysis revealed that pancreatic isoamylase activity, both in serum and urine of newborn infants, was quite low and increased gradually with age (Figure 5). This may reflect the postnatal development of exocrine function in the pancreas (38–40).

Serum and urinary isoamylases of normal adult persons consist of two major isoenzymes (amylase-1 and -3) with high amylase activity and two to three minor ones with low amylase activity. One of the major isoenzymes (amylase-1) has the same electrophoretic mobility as does pancreatic isoamylase; the mobility of the other (amylase-3) is the same as for salivary isoamylase. The pattern of serum amylase isoenzymes was classified into three densitometric types: (a) amylase activity of the pancreatic type is higher than that of salivary type, (b) amylase activity of pancreatic and salivary type is the same, or (c) amylase activity of salivary type is higher than that of pancreatic type. For normal adults, the activity of the pancreatic-type isoenzyme averaged 52.3% of the total. Although the isoenzyme patterns in serum and urine were qualitatively the same, the proportion of amylase activities of isoenzymes in serum and urine from the same person differed: amylase activity of pancreatic-type isoamylases was always higher than that of the salivary type in the urine of almost all of normal adults. This may be the result of higher renal clearance of pancreatic type amylase, as shown by Kamaryt (41) and Duane et al. (42), but the same results were obtained even in patients with renal failure. From these findings, it could be said that the smaller proportion of pancreatic isoenzyme in urine may indicate an insufficiency of pancreatic exocrine function when the value for total urinary amylase activity is normal. However, because the proportion of
Amylase isoenzyme activity in the serum of normal persons differs among individuals, serial examinations of serum amylase isoenzymes are necessary in clinical practice. The importance of serial determination of serum amylase isoenzymes is shown by data for a patient (Figure 6). Total serum amylase activity was at the low normal level and did not change during the period of observations, although the patient complained of severe abdominal pain (Figure 6A). Analysis of serum amylase isoenzymes revealed that pancreatic-type isoamylase, which accounted for only 17% of the total serum amylase activity before the attack of abdominal pain (Figure 6B-1), had increased by as much as 50% during the attack (Figure 6B-2). From these changes in the proportion of pancreatic-type isoamylases, the patient was diagnosed as having suffered a relapse of chronic pancreatitis. Because the pattern of amylase isoenzymes in serum obtained during the attack (Figure 6B-2) showed the same pattern as in normal persons (Figure 5B), one might well have overlooked the relapse of pancreatitis if serial determinations of serum amylase isoenzymes had not been undertaken.

Although the serum and urinary amylase isoenzymes of more than 98% of normal persons consisted of two major isoenzymes and two to three minor ones, four different patterns of amylase isoenzymes were observed, as exemplified in Figure 7. Of normal persons, 0.23% had an isoenzyme with slower mobility than amylase-1. We call this rare isoenzyme “amylase-1s” for the present. In the next three patterns, amylase activity of amylase-2, one of the minor components, was increased up to the same level as amylase-1 or -3; 0.96% of these normal subjects showed decreased amylase activity of amylase-3, 0.07% showed decreased activity of both amylase-1 and -3, and 0.49% showed high activity of amylase-1 -2, and -3. It is not clear whether amylase-1s is the same one as those called “Amy SE-1” by Boettcher and De La Lande (43), or “duplicated pancreatic amylase isoenzyme” by Kamaryt and Laxova (7, 44) and Vacikova and Blochova (45), or “Amy B and C” by Merritt et al. (46), or “P1” by Benjamin and Kenny (22). The prevalence of amylase-1s is 0.23%, which is very low as compared with the 16.1% prevalence of duplicated P reported by Kamaryt and Laxova (7, 44), the 10.5% in Caucasian-Americans by Merritt et al. (46), the 10% by Boettcher and De La Lande (43), or the 5.3% reported by Vacikova and Blochova (45). Are these differences in prevalence due to racial differences or to the method of electrophoresis? Kamaryt and Laxova (7, 44) and Vacikova and Blochova (45) found the duplicated P isoenzyme by use of agar gel electrophoresis. Boettcher and De La Lande (43) and Merritt et al. (46) used polyacrylamide gel slab electrophoresis, whereas our amylase-1s was found by electrophoresis on thin layers of polyacrylamide. We tentatively conclude that the difference in prevalence of this rare isoamylase is not attributable to the method of electrophoresis but is racial in origin.

Results of family studies (47) indicate that all of the four patterns of amylase isoenzymes shown in Figure 7 are under genetic control, so it is important to consider not only diseases but also genetic background before coming to a conclusion when amylase isoenzymes are used for clinical medicine.

In summary, we could separate the isoenzymes of amylase in serum, urine, and extracts of tissue homogenates by simple electrophoresis on thin-layer polyacrylamide gel. Such analysis of amylase isoen-
zyme is useful as a diagnostic tool for various diseases. However, it is always necessary to consider the conditions of samples and the genetic background carefully before coming to a conclusion.

References


